Vaccine 32 (2014) 2337–2344

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Development of a Rift Valley fever virus viremia challenge model in sheep and goats

Hana M. Weingartl[a,b,*], Myrna Miller[c], Charles Nfon[a], William C. Wilson[d]

[a] National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, Manitoba, Canada
[b] Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada
[c] University of Wyoming, Laramie, WY, USA
[d] Arthropod-Borne Animal Diseases Research Unit, ARS, USDA, Manhattan, KS, USA

ARTICLE INFO

Article history:
Received 19 October 2013
Received in revised form 6 February 2014
Accepted 12 February 2014
Available online 12 March 2014

Keywords:
Rift Valley fever
Viremia
Virus isolation
Real-time RT-PCR

ABSTRACT

Rift Valley fever virus (RVFV), a member of the family *Bunyaviridae*, causes severe to fatal disease in newborn ruminants, as well as abortions in pregnant animals; both preventable by vaccination. Availability of a challenge model is a pre-requisite for vaccine efficacy trials. Several modes of inoculation with RVFV ZH501 were tested on goats and sheep. Differences in development of infectious viremia were observed between animals inoculated with RVFV produced in mosquito C6/36 cells compared to Vero E6 cell-produced inoculum. Only C6/36-RVFV inoculation led to development of viremia in all inoculated sheep and goats. The C6/36 cell-produced RVFV appeared to be more infectious with earlier onset of viremia, especially in sheep, and may also more closely represent a field situation. Goats were somewhat more resistant to the disease development with lower and shorter infectious virus viremia, and with only some animals developing transient increase in rectal temperature in contrast to sheep. In conclusion, a challenge protocol suitable for goat and sheep vaccine efficacy studies was developed using subcutaneous inoculation of 10^7 PFU per animal with RVFV ZH501 produced in C6/36 cells.

1. Introduction

Rift Valley fever virus (RVFV) is a member of the family *Bunyaviridae*, genus *Phlebovirus*. This zoonotic arbovirus, endemic to Africa and Arabian Peninsula, causes acute disease in newborn ruminants with up to 100% fatality rate, as well as acute disease in pregnant animals resulting in abortion storms. Naturally infected animals develop high viremia sufficient to infect the arthropod vector, even if the infection is inapparent. The economically important affected species include sheep, goat, cattle and camel, with the primary route of infection being mosquito bites. Humans can be infected by mosquito bites, and importantly also by exposure to blood and tissues of infected ruminants during slaughter, necropsy or while assisting aborting animals [1,2].

Although the disease and development of viremia in ruminants is preventable by vaccination, and ruminant vaccination is recommended to protect human population from RVFV infections, the number of RVFV vaccines in use is limited [3,4]. Availability of a reliable challenge model is a pre-requisite for future vaccine development, registration and licensing. The clinical outcome of experimental infections of ruminants is dependent on RVFV strain used for inoculation, animal breed and age, as well as individual animal variations. The dramatically different clinical outcome of experimental infections makes vaccine evaluation difficult. There are currently two challenge models employed for vaccine efficacy trials in ruminants, both possessing inherent problems [5–8]. The abortion model is cumbersome with synchronization of the pregnancy and scheduling of high biosecurity facilities. The drawback of a viremia model can be a lack of consistency, as not all experimentally inoculated animals may develop detectable viremia [5,9–11], although sensitivity of detection may had been also an issue. For example Yedloutschnig et al. [12,13] titrated the virus inoculum for sheep and cattle inoculations in Vero cells, but used more sensitive intraperitoneal inoculation of 4–6 days old mice to detect viremia in the infected ruminants. Currently, RNA detection is used

* Corresponding author at: National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, 1015 Arlington Street, Winnipeg, MB R3E 3M4, Canada. Tel.: +1 204 789 2027; fax: +1 204 789 2038.
E-mail address: hana.weingartl@inspection.gc.ca (H.M. Weingartl).

http://dx.doi.org/10.1016/j.vaccine.2014.02.066
0264-410X

to compensate for the lower sensitivity of virus isolation in cell culture.

Different age animals were used in previous studies, ranging from one-day-old lambs to several years old adults. Our experimental target age was 3–4 months, when sheep and goats are usually vaccinated on farms.

Virus doses used in the inocula in the reviewed reports were of a wide range, titrated on different substrates, and therefore difficult to directly compare. Often, viremia outcome was not in correlation with the dose. This may be possibly related to individual and breed variations, and to a low number of animals used in most studies (two to four animals for the same route and dose). Overall it appears that lower doses lead to somewhat later development of viremia, delaying its detection from day one to 2–3 days post inoculation. An intraperitoneal route of inoculation was often used in the early experiments, while more recently subcutaneous route is used in majority of studies. Additional or alternative routes have been also tested, such as mucosal, intravenous, or intradermal inoculation [5–13,15,18,19].

There are very few, older publications on the experimental inoculations of goats, suggesting that the duration of viremia may be shorter than in sheep: between 1 and 3 dpi, both days inclusive [16,17]. There is one report currently published on vaccine safety in goats [20], but there are no reports on vaccine efficacy studies in goats; the second most susceptible ruminant species to Rift Valley fever virus. Recently, our group started to work on the experimental infections of goats [21], as vaccine immunogenicity, safety and efficacy testing in this target species may be also required.

The aim of this study was to develop a viremia model in goats and sheep of vaccine age (3–4 months) suitable for vaccine efficacy studies. Up to this point, the RVFV inocula were prepared using a substrate of mammalian origin, e.g. sheep and mouse serum, tissues from infected sheep and mice, or mammalian-origin cell cultures, most frequently Vero and BHK cells, regardless of the origin of the virus isolate [10–18]. To improve the infection model, virus propagated in *Aedes albopictus* cells (C6/36) was compared to virus propagated in mammalian cell line Vero E6. The outcomes of the experimental infections resulting in a proposed RVFV challenge model for vaccine evaluation are discussed.

2. Materials and methods

2.1. Cells and viruses

Vero E6 and C6/36 cells were obtained from American Tissue Culture Collection. Vero E6 cells were maintained in DMEM/10% fetal bovine serum (Wisent) at 37 °C in 5% CO_2 incubator. The C6/36 cells were maintained in 47% ESF-921 (Expression Systems)/47% EMEM/2.5% fetal bovine serum (Wisent)/2.5% HEPES (25 mM final)/1% sodium pyruvate (1 mM final)(Sigma–Aldrich) at 28 °C in sealed flasks (Corning).

RVFV, strain ZH501 [22], was kindly provided by Dr. Heinz Feldmann (National Microbiology Laboratory, Winnipeg). Passage no. 2 was transferred from National Microbiology Laboratory to National Centre for Foreign Animal Disease (NCFAD). The virus was then expanded in Vero E6 cells once, and NCFAD passage two was used in inoculations with RVFV-Vero E6. NCFAD passage two was used to prepare the RVFV-C6/36 stock for animal inoculations. The virus was sequenced at passage two in Vero E6 cells, and then at passage four (used for animal infections), and also at passage two in C6/36 cells (used in animal infections). All three genomic sequences were considered identical, also with the sequence published in GenBank for RVFV-ZH501. Both virus stocks were characterized on genomic and on protein level [21,23].

Single virus stock prepared either in Vero E6 cells or C6/36 cells was used for all respective animal inoculation experiments.

2.2. Virus plaque titration

The virus stocks, inocula and sera were plaque-titrated as follows: 400 μl/well of ten-fold serially diluted samples in DMEM were incubated on confluent monolayers of Vero E6 cells in 12 well plates in triplicates at 37 °C in 5% CO_2 for 1 h. The inoculum was replaced by 1.75% carboxymethyl cellulose (Sigma–Aldrich) in DMEM/0.3% (Wisent) supplemented with 25 mM HEPES (Sigma–Aldrich)/100 μg/ml of Streptomycin/100 IU/ml of Penicillin (Wisent), and incubated for 4 days at 37 °C, 5% CO_2. Formalin (10%) fixed plates were stained with crystal violet (0.5% (w/v) in 80% methanol in PBS), and virus titer determined in PFU/ml.

2.3. Virus detection

Serum samples were simultaneously analyzed by virus isolation using plaque titration as described above to determine viremia, and by real time RT-PCR to determine virus RNA load.

2.4. One-step real-time RT-PCR

RNA isolation from serum using TriPure (Roche Diagnostics) according to manufacturer's instructions was followed by one-step real time RT-PCR targeting the L gene [9].

2.5. Antibody detection

Virus neutralizing antibodies were determined by plaque reduction neutralization assay as described previously [21] on Vero E6 cells using virus produced in Vero E6 cells.

2.6. Animals

All animals in this study were 4 months old at the time of inoculation. Sheep (Suffolk cross, Rideau Arcott cross, Ile-de-France cross with Rideau Arcott) and goats (Alpine-Boer cross) were obtained from breeders in Manitoba. All animal manipulations were approved by the Animal Care Committee of the Canadian Science Centre for Human and Animal Health in compliance with the Canadian Council on Animal Care guidelines (Animal Use Documents #C-08-007, #C-09-004, #C-10-001, #C-11-011). The work with infected animals was performed under containment level 3 conditions (zoonotic BSL-3 Ag).

2.7. Experimental design

Animals were acclimatized for two weeks prior to inoculation and inoculated subcutaneously (SC) with 1 ml of RVFV (ZH501) into the right side of the neck, and if applicable re-inoculated SC or intravenously (IV) depending on the inoculation group. Two doses were compared: "low" dose of 10^5 PFU per animal and "high" dose of 10^7 PFU per animal. Rectal temperatures were taken for three days following arrival of the animal to the facility and for minimum of five days prior to inoculation, and daily during the first week post inoculation. Except for the first group (sheep group A; see below), blood was collected daily for up to 6 or 7 days post inoculation (dpi). At this time point animals were either euthanized to determine virus presence in liver and spleen, or were kept up to 35 dpi for serum production, and bled weekly to follow antibody development (not reported in this manuscript). Overview of the inoculation groups is provided in Table 1. Where it was possible to group animals to compare two experimental approaches, Student's

Table 1
Overview of animal inoculation groups.

	No. of animals	Breed	Inoc.	Dose $\log_{10}$	Route	1 dpi re-inoculation	Viremia duration	RNA detection	Viremia
Sheep								[a]	[a]
S-A	8	SX	Vero	5	SC	–	ND	8/8	8/8
S-B	4	AR	Vero	5	SC	–	3 days	3/4	2/4
S-C	4	AR	C6	5	SC	–	2	4/4	4/4
S-D	4	AR	Vero	7	SC	–	1	4/4	4/4
S-E	8	AR	C6	7	SC	–	4	8/8	8/8
S-F	4	AR	C6	7	SC	SC	4	4/4	4/4
S-G	4	RX	C6	7	SC	IV	3	4/4	4/4
Goat									
G-A	4	BX	Vero	5	SC	–	2	4/4	4/4
G-B	4	BX	C6	5	SC	–	2	4/4	4/4
G-C	4	BX	Vero	7	SC	–	3	4/4	4/4
G-D	4	BX	C6	7	SC	–	2	4/4	4/4
G-E	4	BX	Vero	5	SC	IV	2	4/4	4/4
G-F	4	BX	C6	5	SC	IV	3	4/4	4/4
G-G	4	BX	C6	7	SC	SC	2	4/4	4/4

SX, Suffolk cross; AR, cross of Arcott and Rideau; RX, Rideau cross with Ile de France (some animals were AR, but no apparent differences were observed among the animals in development of viremia); BX, Boer cross. Duration of viremia as determined for infectious virus.

[a] Number of animals tested positive/total number of animals in the group.

t-test was performed. A P value <0.05 was considered statistically significant.

Sheep: Group **S-A**: eight animals (Suffolk cross) were inoculated with 10^5 PFU of RVFV prepared in Vero E6 cells. In this pilot trial, blood was collected at 3, 5 and 7 dpi. Group **S-B**: four animals (Rideau Arcott cross) were inoculated with 10^5 PFU of RVFV Vero E6 stock. Group **S-C**: four animals (Rideau Arcott cross) were inoculated with 10^5 PFU of RVFV C6/36-stock. Group **S-D**: four animals (Rideau Arcott cross) were inoculated with 10^7 PFU of Vero E6 stock. Group **S-E**: eight animals (Rideau Arcott cross) were inoculated with 10^7 PFU of C6/36-stock in two separate trials. Group **S-F**: four animals (Rideau Arcott cross) were inoculated with 10^7 PFU of C6/36 stock and re-inoculated at 1 dpi SC with the same dose. Group **S-G**: 4 animals (Rideau cross with Arcott or Ile de France) were inoculated with 10^7 PFU of the C6/36 derived virus stock, followed by IV inoculation with the same dose at 1 dpi.

Most of the sheep were euthanized at 6–7 dpi, except for few animals kept for antibody production for 28 dpi. Some of the animals kept for production of antiserum were boosted at 14 dpi.

Goats: All animals were Boer cross in groups of four. Group **G-A** was inoculated with 10^5 PFU of Vero E6 derived RVFV stock. Group B **G-B** was inoculated with 10^5 PFU of C6/36 derived RVFV stock. Group **G-C** was inoculated with 10^7 of Vero E6 derived virus. Group **G-D** was inoculated with 10^7 C6/36 derived RVFV. Group **G-E** was inoculated with 10^5 PFU of Vero E6 RVFV stock, and re-inoculated IV with the same inoculum at 1 dpi. Group **G-F** was inoculated with 10^5 PFU of C6/36 derived RVFV, and re-inoculated IV with the same inoculum at 1 dpi. Group **G-G** was inoculated with 10^7 C6/36 derived RVFV and re-inoculated SC with the same inoculum at 1 dpi.

All goats were kept for four weeks following the inoculation to monitor an antibody development. Serum samples collected at 0, 4, 5, 6, 7, 14, 21 and 28–30 dpi were analyzed for presence of neutralizing antibodies.

3. Results

Differences in susceptibility to RVFV infections were observed between sheep and goats, and also between breeds of sheep. In the first study, conducted in Suffolk-cross sheep, all animals developed viremia at 3 dpi, both by virus isolation and RNA detection when inoculated with 10^5 PFU of virus produced in Vero cells. However, when the Rideau Arcott cross lambs were inoculated via the same route and the same inoculum, only three out of four animals had detectable RVFV RNA in their blood and only two developed viremia (Fig. 1). Subsequently different inoculation approaches were tested to obtain a more reliable viremia model.

Genomic sequences of the inocula were verified prior to the start of the animal inoculations. Concurrently with the infection experiments, characterization on protein level of RVFV generated in Vero E6 cells or the C6/36 was taking place. There was no difference in genome of RVFV generated in Vero E6 cells compared to virus generated in C6/36 cells, including the stock viruses used in experimental inoculations, and the sequences corresponded with sequences published for RVFV ZH501 in Gen Bank. Both viruses had functional NSm and NSs coding genes, as immunoblots of infected cell lysates indicated that all proteins from the M and S segments were expressed. The viruses however differed in protein composition of virions, with the mosquito-cell generated RVFV having an additional large glycoprotein (78 kDa) incorporated into virions [23].

3.1. Viremia

Subcutanous inoculation was used in all primary inoculation. Two doses (10^5 or 10^7 PFU/animal) and two different inocula (prepared either in Vero E6 or in C6/36 cells) were tested. The titer of inoculum was confirmed by back-titration at the time of inoculation, and stayed within 0.5 $\log_{10}$ difference from the targeted dose. In specific groups, attempts were made to increase the viremia by re-inoculation, either by the subcutaneous or by the intravenous route at 1 dpi. A summary of the experimental groups is presented in Table 1.

3.1.1. Sheep

Using the same mode of inoculation as for the Suffolk breed (group **S-A**), the 10^5 PFU dose of Vero E6 produced RVFV in Rideau Arcott cross lambs (group **S-B**) lead to development of viremia only in three out of four animals at 2 dpi. One sheep appeared to be not infected based on detection of viremia and viral RNA in serum. This experiment was conducted concurrently to inoculation with the same dose of virus produced in the C6/36 insect cells. All animals inoculated with the insect cells derived virus developed viremia at 1 and 2 dpi supported by viral RNA detection (group **S-C**, Fig. 1). Subsequently, a dose of 10^7 PFU/animal was tested, again with both, mammalian (group **S-D**) or insect (group **S-E**) cells produced RVFV. At this dose, the Vero E6 inoculum appeared to be even less

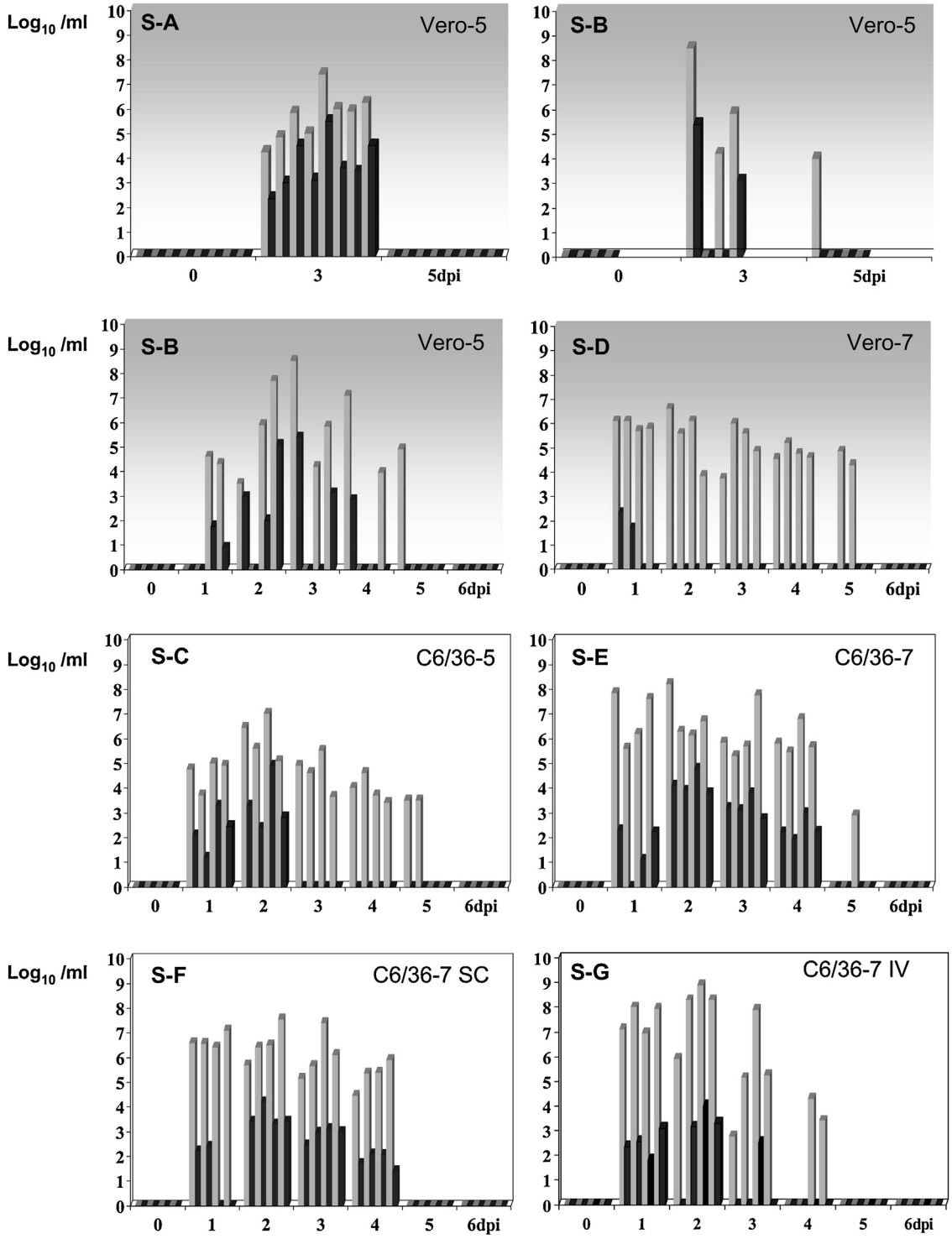

Fig. 1. Comparison of viremia in sheep inoculated with two different doses of viruses prepared either in the Vero E6 or the C6/36 cells in individual animals. The inoculation groups are designated as **S-A** to **S-G**, accordingly to Table 1. Two panels illustrate the difference in viremia between Suffolk-cross sheep (**S-A**) and Arcott-Rideau-cross sheep (**S-B**) inoculated subcutaneously with 10^5 PFU per animal of ZH501 RVFV produced in Vero E6 cells. Second panel designated as **S-B** summarizes viremia in animals inoculated SC with 10^5 PFU of Vero E6 cells-produced RVFV (ZH501) on a daily sampling basis, compared to group Vero-7 (**S-D**) with the dose of 10^7 PFU per animal. Panels **S-C** and **S-E** summarize similarly the inoculations with C6/36 cells-produced virus. Panel **S-F** indicated additional SC re-inoculation and panel **S-G** an additional IV inoculation. Gray columns indicate $\log_{10}$ of viral RNA copy numbers per ml of serum. Black columns indicate $\log_{10}$ of PFU/ml of serum.

effective than the 10^5 PFU dose based on detection of infectious virus, although RNA detection in the serum was higher and of longer duration (Fig. 1, **S-B** versus **S-D**). The most effective infection was achieved by subcutaneous inoculation with 10^7 PFU of C6/36 cells produced virus (group **S-E**), regardless whether the animals were re-inoculated subcutaneously with the same dose or not (Fig. 1, **S-E** and **S-F**). Virus isolation was successful from serum of all inoculated animals at 2, 3 and 4 dpi. Intravenous re-inoculation at 1 dpi appeared to shorten the viremia (Group **S-G**, Fig. 1). The **S-E** model was chosen as a challenge control for efficacy testing of vaccine candidates [24]. Since the RVFV used in the challenge were the aliquots of the same virus stock used for this study, we have added

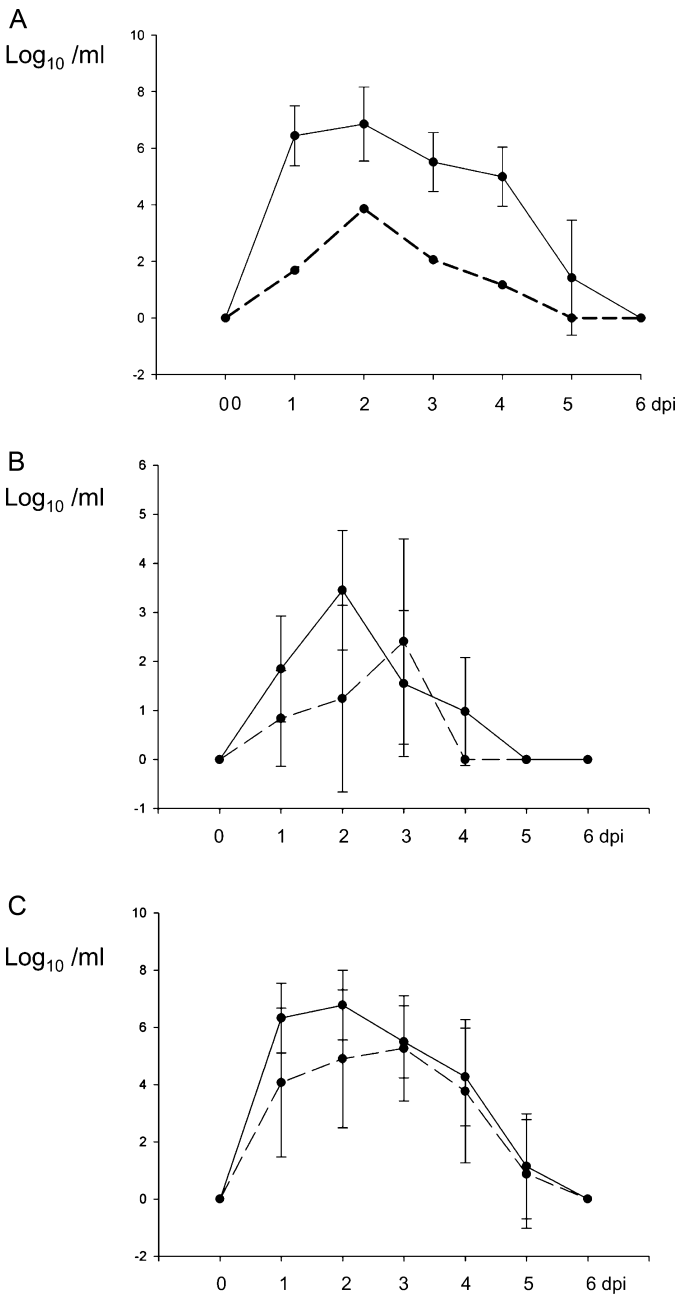

Fig. 2. Summary of viremia development in sheep. (A) RVFV viremia in sheep inoculated with 10^7 PFU of C6/36 cell-produced virus per animal ($n = 8$). Solid line – viral RNA in $\log_{10}$ copy number per ml of serum, dashed line infectious virus viremia in serum in $\log_{10}$ PFU per ml. (B) Comparison of viremia in sheep between groups inoculated with Vero E6-derived virus or with C6/36 cell-derived virus. Viremia in $\log_{10}$ PFU/ml of serum. Solid line inoculum generated in C6/36 cells, dashed line – inoculum generated in the Vero E6 cells. (C) Comparison of viremia in sheep between groups inoculated with Vero E6-derived virus or with C6/36 cell-derived virus. Viral RNA in $\log_{10}$ copy numbers/ml of serum. Solid line inoculum generated in C6/36 cells, dashed line – inoculum generated in the Vero E6 cells.

in Fig. 2 the results from the four challenge control animals to the group to make it statistically stronger ($n = 8$; Fig. 2A). In order to be able to perform at least minimal statistical comparison of the inoculation approaches we have grouped animals inoculated with the Vero E6 produced virus into one group ($n = 16$), and the animals inoculated with the C6/36 produced virus into a second group ($n = 20$). Viremia was significantly higher in lambs inoculated with the insect cells produced virus at days 1 and 2 post inoculation ($P = 0.03$ and $P = 0.01$, respectively) (Fig. 2B). Correspondingly, the RVFV RNA levels in serum were also higher in the insect cell virus inoculated animals (days 1 and 2 post inoculation; $P = 0.004$ and $P = 0.01$ respectively) (Fig. 2C).

3.1.2. Goats

Several inoculation approaches lead to development of viremia in all inoculated Alpine-Boer cross goats, although goats were in general less sensitive to RVFV infection then the sheep based on infectious virus titers and duration of the viremia. Subcutaneous inoculation with Vero cells-produced virus lead to development of viremia either at 2 or 3 dpi (groups **G-A** and **G-E**) or between 1 and 3 dpi (groups **G-C**) (Fig. 3) with maximum duration of two days. Interestingly, the low dose of Vero-cell produced virus caused viremia a day later compared to all other inoculation approaches (groups **G-A** and **G-E**)(Fig. 3).

Inoculation with the 10^7 PFU of C6/36-produced virus (groups **G-D** and **G-G**) lead to development of viremia in all animals at the same day (1 dpi), making it easier to evaluate (Fig. 3). One goat in group **G-C** died suddenly between 1 and 2 dpi without apparent clinical signs, and without increase in rectal temperature (at 1 dpi, the temperature was 39.4 °C). There were no pathological changes found on necropsy, and the virus load was not high compared to typical virus titers recovered from serum, liver, spleen or other organs and tissues. Cause of death was therefore considered as unknown, although it cannot be excluded that the animal died due to RVFV infection. Statistical comparison of the detected RVFV RNA levels between goats inoculated with Vero E6-produced virus ($n = 12$) and goats inoculated with C6/36 cells-produced virus ($n = 16$) indicated that the developed viremia was higher with faster onset in animals infected with insect cell-derived virus ($P = 0.002$) (Fig. 4A). When the dose 10^7 PFU/animal of virus of either origin was evaluated separately, the insect-derived virus caused faster onset of the viremia, with the significantly higher RNA levels at 1 dpi ($P < 0.001$) (Fig. 4B).

3.2. Rectal temperatures

Increase in rectal temperature can be used as one of the parameters in challenge studies in sheep to evaluate efficacy of the vaccine candidates, but is unfortunately not applicable for goats.

All RVFV inoculated lambs experienced minimum one or two days of increased rectal temperatures, with no significant differences between individual inoculation approaches (Fig. 5). On the other hand, out of all 28 RVFV inoculated goats only 11 random animals developed increased rectal temperatures for one day.

3.3. Neutralizing antibodies

Although antibody development was not the main focus of the study, due to limited knowledge on RVFV infection in goats, the animals were kept for 28–30 dpi, and serum collected during the animal inoculation experiments was analyzed by plaque reduction neutralization assay. Development of neutralizing antibodies against RVFV in goats is summarized in Fig. 6. Significant difference in antibody titers, related to inoculation dose, was observed at 14 dpi. Animals infected with 10^7 PFU of either Vero E6 or C6/36 cell-produced virus developed at least four-fold higher antibody titers than goats infected with 10^5 PFU, however a continuous gradual increase in antibody titers until the end of the experiment was observed in serum of animals inoculated with the lower dose. Very interestingly, goats infected with high dose of mosquito cell-produced virus experienced a drop in neutralizing titers by 28 dpi, while goats infected with the Vero E6 cell-produced RVFV maintained their antibody levels at 21 dpi also at 28 dpi.

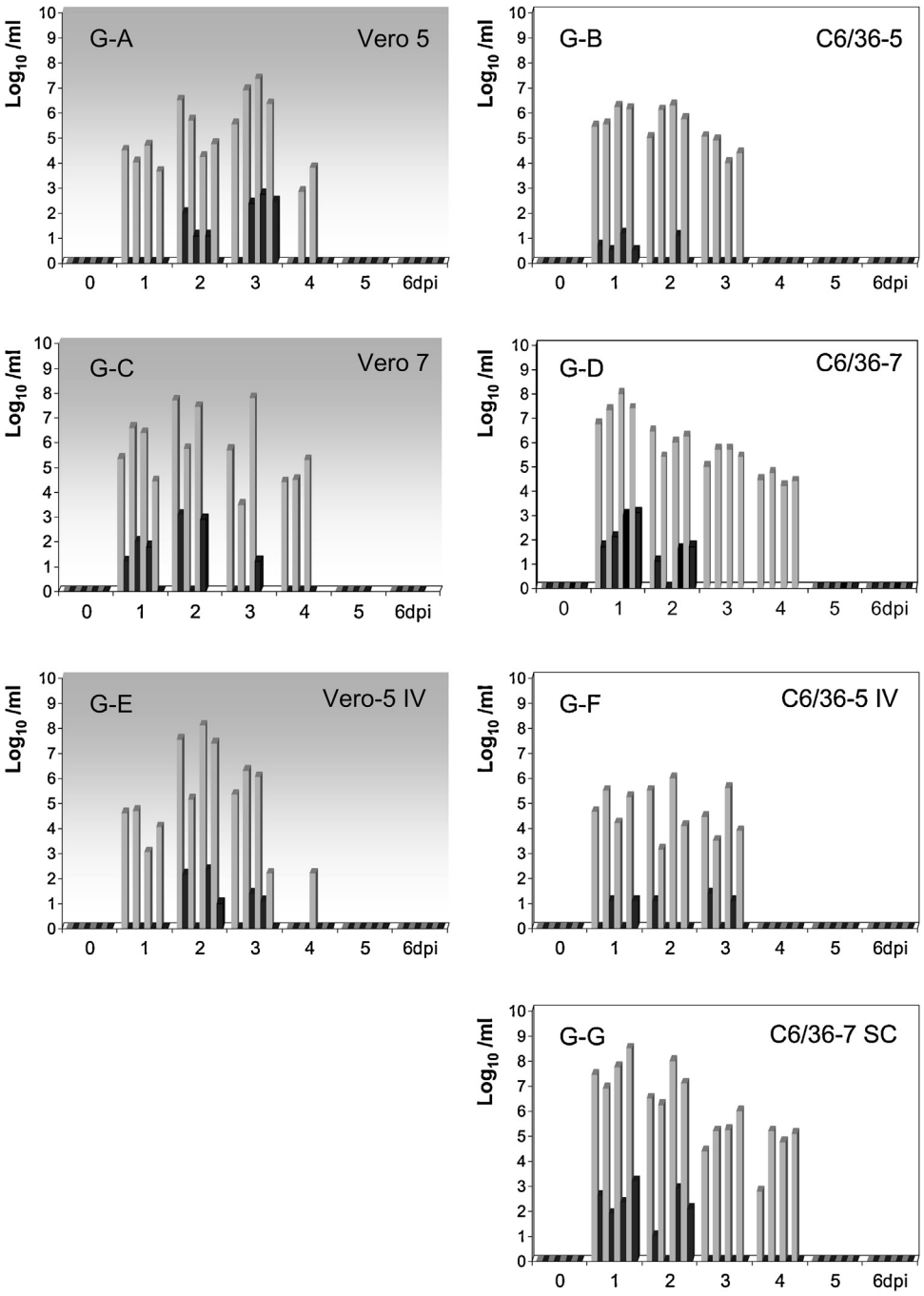

Fig. 3. Comparison of viremia in goats inoculated with two different doses of viruses prepared either in the Vero E6 or the C6/36 cells in individual animals. The inoculation groups are designated as **G-A** to **G-G**, accordingly to Table 1. Panels on the left side summarize inoculations with Vero E6 produced virus inoculated SC with 10^5 PFU/animal (**G-A**), with 10^7 PFU (**G-C**) or with 10^5 PFU followed by IV re-inoculation. The panel on the right side summarize the inoculations with C6/36 derived RVFV (ZH501), using the dose of 10^7 PFU per animal (**G-D**) and followed by SC re-inoculation (**G-G**) or using the dose of 10^5 PFU/animal (**G-B**) and followed by IV re-inoculation (**G-F**). Gray columns indicate $\log_{10}$ of viral RNA copy numbers per ml of serum. Black columns indicate $\log_{10}$ of PFU/ml of serum.

A difference in the onset of antibody response was observed between goats and sheep. While serum samples collected at 4 dpi were all negative, first neutralizing antibodies were detected at 5 dpi in 92.5% of goats, and on day 6 post infection all goats seroconverted. In comparison, only 85% of sheep seroconverted at 6 dpi, with all serum samples collected at 7 dpi being positive for neutralizing antibodies. The antibody titers at 7 dpi for both, goats and sheep were about the same, in range of 20–40, for all the animals.

4. Discussion

The aim of this study was to develop a viremia model in goats and sheep of vaccine age (3–4 months) suitable for vaccine efficacy studies.

Low passage RVFV was used for the animal inoculations as high passage virus in the same cell line may acquire deletions resulting in loss of protein expression, e.g. NSs protein, one of the virulence determinants [25]. In addition, the genomic sequence and protein

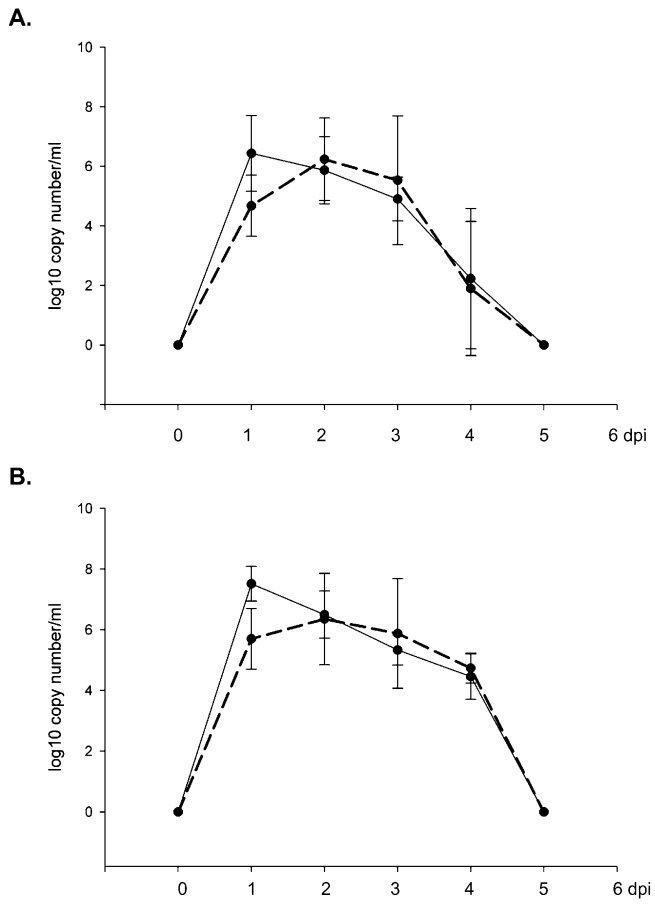

Fig. 4. Comparison of RNA detection in goats. (A) Virus inoculum derived in Vero cells is represented by dashed line, C6/36-produced virus is represented by the full line. (B) Comparison of the two types of inocula used at the 10^7 PFU dose. Virus inoculum derived in Vero cells is represented by dashed line, C6/36-produced virus is represented by the full line.

expression were verified for the virus stock generated in Vero E6 cells as well as for the RVFV stock generated in C6/36 cells [21,23].

Based on the obtained data, both sheep and goats appear to be more sensitive to RVFV challenge using virus produced in C6/36 *A. albopictus* mosquito cells compared to Vero E6 cells when administered subcutaneously. Besides the intuitive reasoning that the use

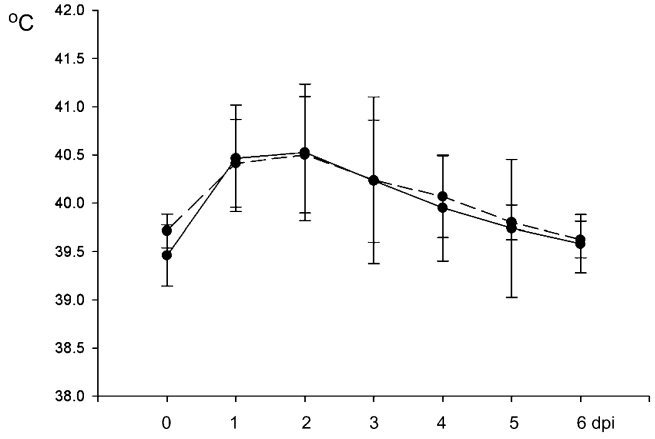

Fig. 5. Rectal temperatures of sheep inoculated with RVFV ZH501. Temperatures of animal inoculated with virus produced in Vero E6 cells are represented by dashed line, temperatures of animals inoculated with C6/36-produced virus are represented by the full line.

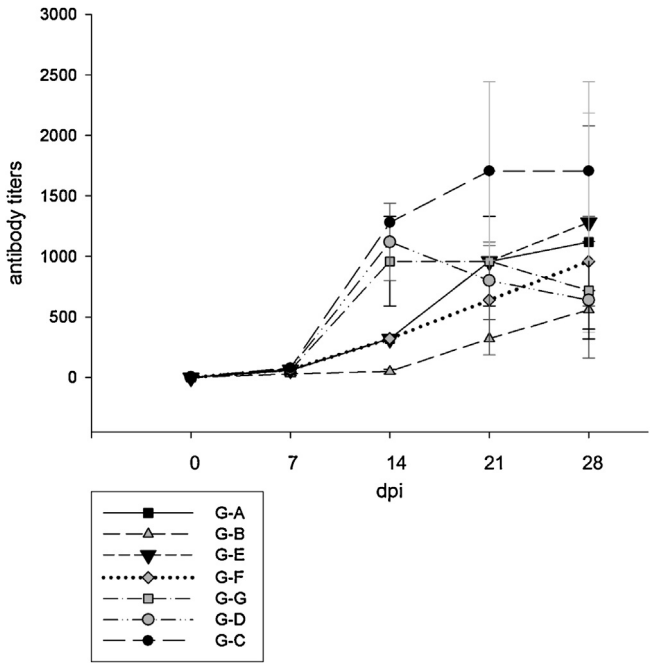

Fig. 6. Antibody development in goats up to 28 dpi. Goats inoculated with RVFV produced in C6/36 cells have gray symbols; goats inoculated with Vero-E6 produced virus have black symbols. Designation of lines and symbols for individual animal inoculation groups is incorporated into the figure.

of mosquito cell derived virus administered subcutaneously more closely mimics the field transmission of RVFV from mosquitoes to ruminants than the use of mammalian derived virus or the IV route of challenge, our previous studies also suggested that the mosquito cell produced virus may be more efficient in initiating the infection via the subcutaneous route. Experimental infection of goats indicated a difference between Vero cell-produced inoculum and the inoculum produced in C6/36 cells at the immune response level [21]. RVFV has been shown to infect monocyte-derived dendritic cells [26]. Current reports on replication of other arboviruses in dendritic cells, the primary target of these viruses in the host skin, indicate that there is indeed a biological difference between virus produced in mammalian cells compared to virus produced in insect cells in terms of virus–host cell attachment, differential activation of the dendritic cells and evasion of innate immune response such as ineffective IFN-type I induction [27–29] resulting in enhanced infectivity of the mosquito-origin virus for mammalian dendritic cells compared to mammalian-origin viruses. RVFV, in addition to presumably different lipid composition of the envelope and different type of glycans on viral glycoproteins, incorporates into the mosquito-cell matured virions also the large 78 kDa protein [23] which could further facilitate the interspecies transmission from mosquitoes to ruminants. We hypothesized that use of insect cell-produced RVFV inoculum administered subcutaneously would lead to consistent and measurable viremia in sheep and goats, representing a suitable model for veterinary vaccines efficacy studies.

On the other hand, use of virus inoculum prepared in mammalian cells administered via mucosal surfaces [30] appears to better mimic human infections acquired through exposure to blood and tissues of ruminants infected with RVFV, and would be well suited for human vaccines efficacy studies.

We have also attempted to increase the viremia with different route of re-inoculation at 1 dpi, in case the early immune response is partially suppressed by initial virus replication. No observed differences may be due to a late administration of a second dose, and the model may be possibly further improved by early re-inoculation within a shorter period than one day, when the first round of virus

replication still temporarily suppresses host immune responses [21].

The shorter duration of viremia in goats compared to sheep was in agreement with previously published data [16,17], and may be possibly accounted to somewhat faster onset of humoral immune response, as one of the species specific factors. Interesting observation was made with regard to shorter duration of antibody levels in goats infected with high dose of mosquito-cell produced virus compared to mammalian-cell produced RVFV, indicating a need for a long term study to evaluate performance of serological diagnostic tests for this species.

In conclusion, the following challenge protocol was determined to be suitable for goat and sheep vaccine efficacy studies: subcutaneous inoculation into the right side of the neck with 10^7 PFU per animal of RVFV ZH501 produced in C6/36 cells.

Acknowledgements

We would like to thank the NML, PHAC and NCFAD, CFIA animal care staff, especially M. Forbes, J. Bernstein, K. Tierney, and C. Nakamura for their help with the animal experiments. The authors would further like to thank S. Zhang, B. Dalman, B. Solylo, E. Weingartl and P. Marszal for the technical assistance. The project was funded in part by a CRTI project RD-06-0138, by CFIA, USDA, ARS CRIS project 5430-32000-005-00D and a U.S. Department of Homeland Security Interagency Agreement HSHQDC-07-X-00982. The contents of this publication are solely responsibility of the authors.

References

[1] Pepin M, Bouloy M, Bird BH, Kemp A, Paweska J. Rift Valley fever virus (*Bunayviridae: Phlebovirus*): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. Vet Res 2010;41:61, http://dx.doi.org/10.1051/vetres/2010033.
[2] Flick R, Bouloy M. Rift Valley fever virus. Curr Mol Med 2005;5:827–34.
[3] The use of veterinary vaccines for prevention and control of Rift Valley fever: memorandum from a WHO/FAO meeting. Bull World Health Organ 1983;61:261–8.
[4] Boshra H, Lorenzo G, Busquets N, Brun A. Rift Valley fever: recent insights into pathogenesis and prevention. J Virol 2011;85:6098–105.
[5] Kortekaas J, Antonis AFG, Kant J, Vloet RPM, Vogel A, Oreshkova N, et al. Efficacy of three candidate Rift Valley fever vaccines in sheep. Vaccine 2012;30:3423–9.
[6] Bird BH, Maartens LH, Campbell S, Erasmus BJ, Erickson BR, Dodd KA, et al. Rift Valley fever virus vaccine lacking the NSs and NSm genes is safe, nonteratogenic, and confers protection from viremia, pyrexia, and abortion following challenge in adult and pregnant sheep. J Virol 2011;85:12901–9.
[7] Dungu B, Louw I, Lubisi A, Hunter P, von Teichman BF, Bouloy M. Evaluation of the efficacy and safety of the Rift Valley fever Clone 13 vaccine in sheep. Vaccine 2010;28:4581–7.
[8] Morill JC, Mebus CA, Peters CJ. Safety and efficacy of a mutagen-attenuated Rift Valley fever virus vaccine in cattle. AJVR 1997;58:1104–9.
[9] Drolet BS, Weingartl HM, Jiang J, Neufeld J, Marszal P, Lindsay R, et al. Development and evaluation of one-step rRT-PCR and immunohisto-chemical methods for detection of Rift Valley fever virus in biosafety level 2 diagnostic laboratories. J Virol Methods 2012 Feb;179(2):373–82, 2012.
[10] Busquets N, Xavier F, Martín-Folgar R, Lorenzo G, Galindo-Cardiel I, del Val BP, et al. Experimental infection of young adult European breed sheep with Rift Valley fever virus field isolates. Vector Borne Zoonotic Dis 2010;10(October (7)):689–96, http://dx.doi.org/10.1089/vbz.2009.0205.
[11] Tomori O. Clinical, virological and serological response of the West African dwarf sheep to experimental infection with different strains of Rift Valley fever virus. Res Vet Sci 1979;26:152–9.
[12] Yedloutschnig RJ, Dardiri AH, Mebus CA, Walker JS. Abortion in vaccinated sheep and cattle after challenge with Rift Valley fever virus. Vet Rec 1981;109:383–4.
[13] Yedloutschnig RJ, Dardiri AH, Walker JS. Rift Valley fever infection in sheep by contact exposure. Contrib Epidemiol Biostat 1981;3:53–9.
[14] Olaleye OD, Tomori O, Fajimi JL, Schmitz H. Experimental infection of three Nigerian breeds of sheep with the Zinga strain of the Rift Valley fever virus. Rev Elev Med Vet Pays Trop 1996;49:6–16.
[15] Easterday BC, Murphy LC, Bennet DG. Experimental Rift Valley fever in lambs and sheep. Am J Vet Res 1962;23:1231–40.
[16] Easterday BC, Murphy LC, Bennet DG. Experimental Rift Valley fever in calves, goats, and pigs. Am J Vet Res 1962;23:1224–30.
[17] Iman ZEI, El-Karamany R, Kasem S. Studies on goats experimentally infected with R.V.F. virus. J Egypt Public Health Assoc 1978;LIII:273–80.
[18] Fagbami AH, Tomori O, Fabiyi A, Isoun TT. Experimental Rift Valley fever in West African Dwarf sheep. Res Vet Sci 1975;18:334–5.
[19] Ross TM, Bhardwaj N, Bissel SJ, Hartman AL, Smith DR. Animal models of Rift Valley fever virus infection. Virus Res 2012;163:417–23.
[20] Kamal SA. Pathological studies on postvaccinal reactions of Rift Valley fever in goats. Virol J 2009;6:94, http://dx.doi.org/10.1186/1743-422X-6-94.
[21] Nfon CK, Marszal P, Zhang S, Weingartl HM. Innate immune response to Rift Valley fever virus in goats. PLoS Negl Trop Dis 2012;6:e1623, http://dx.doi.org/10.1371/journal.pntd.0001623.
[22] Rossi CA, Turell MJ. Characterization of attenuated strains of Rift Valley fever virus. J Gen Virol 1988;69:817–23.
[23] Weingartl HM, Zhang S, Marszal P, McGreevy A, Burton L, Wilson WC. Rift Valley fever virus incorporates the 78 kDa glycoprotein into virions matured in mosquito C6/36 cells. PLoS ONE 2014;9:e87385, http://dx.doi.org/10.1371/journal.pone.0087385, eCollection 2014.
[24] Weingartl HM, Nfon CK, Zhang S, Marszal P, Wilson WC, Morill JC, Bettinger GE, Peters CJ. Efficacy of recombinant Rift Valley fever virus MP-12 with NSm deletion as a vaccine candidate in sheep. Vaccine 2014, http://dx.doi.org/10.1016/j.vaccine. 2013.12.064 [epub ahead of print].
[25] Moutailler S, Roche B, Thiberge JM, Caro V, Rougeon F, Failloux AB. Host alternation is necessary to maintain the genome stability of Rift Valley fever virus. PLoS Negl Trop Dis 2011;5:e1156, http://dx.doi.org/10.1371/journal.pntd.0001156.
[26] Lozach PY, Kuhbacher A, Meier R, Mancini R, Bitto D, Bouloy M. DC-SIGN as a receptor for phleboviruses. Cell Host Microbe 2011;10:75–88.
[27] Klimstra WB, Nangle EM, Smith MS, Yurochko AD, Ryman KD. DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. J Virol 2003;77:12022–32.
[28] Shabman RS, Morrison TE, Moore C, White L, Suthar MS, Hueston L. Differential induction of type I interferon responses in myeloid dendritic cells by mosquito and mammalian-cell-derived alphaviruses. J Virol 2007;81:237–47.
[29] Silva MC, Guerrero-Plata A, Gilfoy FD, Garofalo RP, Mason PW. Differential activation of human monocyte-derived and plasmacytoid dendritic cells by West Nile virus generated in different host cells. J Virol 2007;81:13640–8.
[30] Smith DR, Bird BH, Lewis B, Johnston SC, McCarthy S, Keeney A, et al. Development of a novel nonhuman primate model for Rift Valley fever. J Virol 2012;86:2109–20.